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Peculiarities of the Regulation of Fermentation and Respiration in the Crabtree-Negative, Xylose-Fermenting Yeast *Pichia stipitis*

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ABSTRACT

The respiration of *Pichia stipitis* was not repressed by either high concentrations of fermentable sugars or oxygen limitation. Fermentation was not induced by high sugar concentrations, but was inactivated by aerobic conditions. The activity of pyruvate dehydrogenase was constitutive. In contrast, pyruvate decarboxylase, alcohol dehydrogenase, and aldehyde dehydrogenase were induced by a reduction in the oxygen tension. It was demonstrated that in *P. stipitis* , the pyruvate decarboxylase is not induced by a signal from glycolysis. Contrary to *Saccharomyces cerevisiae*, the pyruvate decarboxylase was not inhibited by phosphate.

Index Entries: *Pichia stipitis*; PDC; PDH; ADH; aldehyde dehydrogenase.

INTRODUCTION

The yeast *Pichia stipitis* converts xylose to ethanol with nearly maximal yield and relatively high rates in comparison to other xylose-fermenting yeasts. However, the rates are rather low in comparison to the glucose fermentation capacity of *Saccharomyces cerevisiae*. This is one of the reasons why *P. stipitis* has not yet been used for the production of ethanol from xylose on an industrial scale *(1,2)*.

Attempts were made to overcome this disadvantage of *P. stipitis* by searching for optimal fermentation conditions, but the results did not lead to substantial improvement *(3)*. A solution could be attained by manipulating the genes of *P. stipitis* coding for enzymes involved in carbohydrate metabolism. The xylose catabolism enables the cells to divide frequently under aerobic conditions. This quick growth is severely retarded under semiaerobic conditions. This retardation does not appear to be caused by a redox imbalance in the cell owing to the reduced respiration, since no xylitol is produced and the decrease in growth rate is also found in glucose growing cells *(4,5)*. Rather, it seems to be the result of a decrease in the metabolic flux through the several steps of the xylose assimilation pathway and glycolysis.

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Only little is known about the regulation of the appropriate steps. It was shown that no fermentation occurs under aerobic conditions in medium containing 3% xylose *(6)*. This result suggested that *P. stipitis* is a Crabtree- negative species *(7)*. In *Candida shehatae*, a species closely related to *P. stipitis (8)*, the activity of alcohol dehydrogenase (ADH) during oxygen limitation is much higher than under aerobic conditions *(9)*. The activity of pyruvate decarboxylase (PDC) of *P. stipitis* is strongly influenced by the oxygen concentration in the medium *(10)*. In the Crabtree-positive species *S. cerevisiae*, detailed knowledge exists about the induction mechanism of PDC. At least it is triggered by distinct metabolic signals that originate from the glycolysis *(11)*. However, there is no experimental evidence for the mode of activation of PDC in *P. stipitis*.

The investigations described here deal with the metabolic regulation at the branching points between respirative and fermentative metabolism, including the regulation mechanism for pyruvate dehydrogenase (PDH), PDC, aldehyde dehydrogenase (AldDH), and ADH. An essential aspect of our research was the comparison of the regulation in the Crabtree-negative yeast *P. stipitis* with that described for *S. cerevisiae*.

MATERIALS AND METHODS

Materials

Yeast Strains

P. stipitis NRRL Y-11544 (CBS 5776), kindly provided by C. P. Kurtzman, was used in all experiments. *S. cerevisiae* ATCC 24858 was used as a reference strain for the examinations of the inhibition of PDC by phosphate.

Media

The cells were grown in a mineral salt medium according to ref. *12*, supplemented with 6.7 g/L yeast nitrogen base w/o amino acids (Difco). If not otherwise stated, 30 g/L glucose or xylose or 20 g/L ethanol were added as described below.

Aerobic and Semiaerobic Growth

Aerobic and semiaerobic growth conditions were achieved by varying the volume of the media *(13)* and the speed of shaking. For aerobic growth, the cells were incubated at 25°C and 200 rpm on a gyratory shaker in a Fernbach flask containing 100 mL media. The cells were incubated up to an OD at 623 nm of 1.0. No ethanol was formed below this optical density, indicating a respirative mode of growth.

For semiaerobic growth, the cells were incubated at 25°C and 100 rpm in a 1-L Erlenmeyer flask containing 250 mL media up to an OD of approx 7.0. Under these conditions, the yield of ethanol was estimated to be approx 0.45 g ethanol/g sugar, indicating a preferential fermentative mode of metabolism.

Shift Experiments

The oxygen shift experiments were performed in a Braun Biostat E 1 fermenter, containing 5 L of media at 25°C. The pH was held constant at 5.0 using 1*M* NaOH. The fermenters were inoculated with 50 mL of aerobically grown cells that were grown on the same carbon source as in the fermenter. The oxygen tension was held constantly at 80% by varying both the rotating speed between 300 and 500 rpm and

the aeration rate. The cells were grown under these conditions until they reached an OD of about 1.0, which was achieved within 8–10 h. At higher cell densities, the oxygen tension started to decrease and caused irregular conditions. To shift the oxygen tension from 80 to 20% or to 0%, the culture was flushed with nitrogen. The shift was achieved within 30 s. After a shift to 20%, the oxygen tension was regulated by varying the aeration rate. After the shift to 0%, the cells were further gassed with nitrogen to produce anaerobic conditions. The rotation speed was held constant at 200 rpm in both cases.

For glucose induction experiments, the fermenter was inoculated with an aerobic culture grown in ethanol and incubated until the cells reached an OD of 0.6. This was reached within 10 h. After this, glucose was added to a final concentration of 3%. The oxygen tension was held constant at 80%. However, this was only possible for a maximum of 4 h. To estimate the enzyme activities and the concentration of glycolytic metabolites of the cells grown in ethanol, additional yeasts were tested that were grown with ethanol to an OD of about 1.0, to avoid systematic errors owing to the inherent low dry weight and protein concentration.

Enzyme Assays

Samples were taken immediately prior to the oxygen shift (aerobic activity) or to the addition of glucose to cells grown in ethanol. After the oxygen tension shift or the addition of glucose, samples were taken at the times described below (*see* Results). The sample volume was about 100 mL. The cell suspension was quickly cooled by transferring to a tube that had been kept in ice. The crude extracts were prepared from this sample as reported *(14)*. The crude extract buffer was supplemented with the protease inhibitors phenylmethanesulfonyl fluoride, leupeptin, and pepstatin A *(15)*. The samples were stored at 4°C until the measurements were performed, which was within 9 h after the preparation of the crude extract. Within this time, no significant decrease of the activities of the tested enzymes was found. The activities of PDC and PDH were determined as described elsewhere *(15,16)*. For the measurements of PDH, the crude extract was purified prior to the assay using a Sephadex 50 column equilibrated with crude extract buffer. The reaction was initiated using pyruvate instead of Coenzyme A. ADH was assayed with 0.6*M* ethanol and 2.5 m*M* NAD^+ in pyrophosphate-semicarbazide buffer containing 75 m*M* $Na_4P_2O_7$, 21 m*M* glycine, and 2.5 g/L semicarbazide. The pH of the buffer was adjusted to 8.7 using 5*M* NaOH. The AldDH activity was assayed with benzaldehyde as substrate *(17)*. Acetaldehyde was also tested, but the activity was found to be nearly the same as benzaldehyde. All measurements were performed in a Beckmann DU 64 photometer or in a Pharmacia LKB Biochrom 4060 photometer.

The enzyme activities are expressed in U/mg protein. One unit (U) of enzyme activity is defined as the ability to convert 1 μmol of substrate/min at 30°C. The protein concentration was determined by the method of Lowry *(18)* using bovine serum albumin as a standard.

Determination of Kinetic Parameters of PDC and PDH

The kinetic parameters of PDC were estimated in cells grown semiaerobically; those of PDH were determined in aerobically grown cells. The behavior of the enzymes was determined by sampling the activity in the crude cell extract at several concentrations of pyruvate (*see* Results). Inhibition of PDC by phosphate was inves-

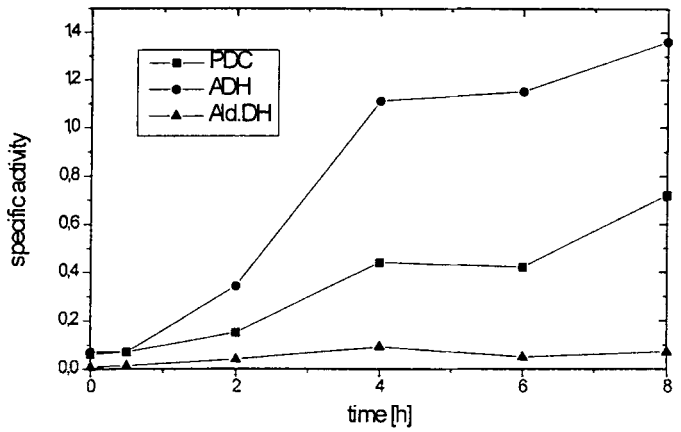

Fig. 1. Specific activities of PDC, ADH, and AldDH of glucose growing cells after a shift of the oxygen tension from 80 to 0%.

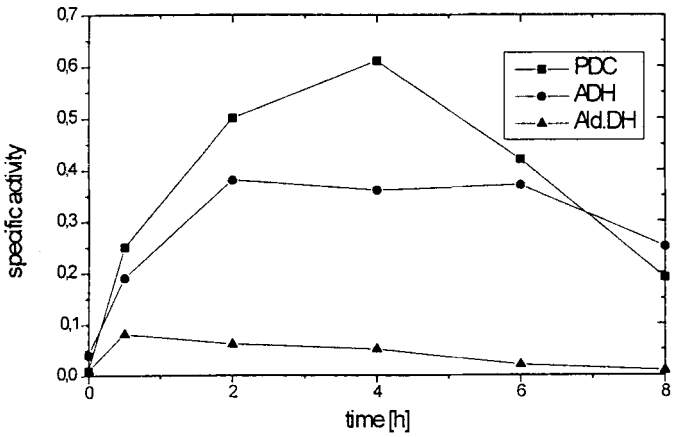

Fig. 2. Specific activities of PDC, ADH, and AldDH of glucose growing cells after a shift of the oxygen tension from 80 to 20%.

tigated in Na phosphate buffer at pH 6.8. As a reference for the inhibition by phosphate, the PDC of *S. cerevisiae* ATCC 24858 pregrown on 2% glucose was measured.

The inhibition of PDH by NADH was estimated against NAD^+ as substrate with 4 m*M* pyruvate. The parameters were calculated as reported *(19)*.

Determination of Glycolytic Metabolites

The samples were prepared and analyzed as described *(14)*. The extracts were stored at –70°C until analyzed in <24 h.

Measurements of Respirative and Fermentative Capacities

The cells were pregrown aerobically or semiaerobically with 6% glucose or xylose, harvested by centrifugation, washed with 50 m*M* Sørensen phosphate buffer (pH 5.5), and diluted with the buffer to an OD of 2.0. This suspension was used for the estimation of respirative and fermentative activities and dry weight.

To determine the activities of anaerobically cultivated cells, the cells were pregrown in a fermenter at a constant aeration rate and stirrer speed (300 rpm) with an initial 3% glucose. When the oxygen tension fell to zero, the amount of consumed glucose was added to restore the concentration to 3%, and the fermenter was gassed with nitrogen for 14 h. The activities were determined using standard Warburg manometers. All manometric measurements were made at 28°C, and lasted 1 h.

Analytical Procedures

Concentrations of sugar and ethanol were determined by HPLC with an Aminex Fermentation Monitoring column (Bio-Rad, Hercules, CA) using RI detector (Melz) with methanol as an internal standard. Dry cell weights were determined gravimetrically from samples containing 10 mL of cell suspension. The samples were harvested by centrifugation, washed twice with distilled water, and incubated at 105°C until they maintained a constant weight. In all cases, three or four measurements were made.

RESULTS

Respirative and Fermentative Activity

The rates of O_2 consumption and CO_2 formation were measured in correlation to different sugar concentrations and aeration conditions over a 60-min period. The cells were precultivated aerobically and semiaerobically on a high level (6%) of sugar, and then quickly transferred to Warburg vessels. No significant differences were found for sugar concentrations from 1.5 to 5.2%.

The respiratory quotients of cells pregrown aerobically on glucose or xylose were between 1.1 and 1.2 in every case, the Qo_2 values were between 81 and 84, and the Qco_2 values were between 93.6 and 99. This proves that no fermentation took place as a result of the high sugar concentrations.

The initial RQ value 1.65 of cells precultivated semiaerobically on 6% glucose indicated that the cells were fermenting. However, the RQs decreased, independently of the sugar concentration, within 30 min to values between 1.2 and 1.0, which were comparable to the RQs obtained under aerobic conditions. The Qo_2 remained constant over the period of the test at 120.0–130.0, which was even higher than in cells cultivated aerobically. The Qco_2 decreased from in the mean 189.4 to 156.9. Similar results were obtained with cells that were precultivated on xylose with the exception that the initial value of the RQ was 1.4. The Qco_2 of these cells decreased from 186.0 to 149.6 within 30 min.

In cells that were precultivated for 14 h under anaerobic conditions, the values of the O_2 consumption and the CO_2 production were only 50–60% of that obtained under semiaerobic conditions. The lower values may be owing to an enhanced number of unviable cells, caused by the anaerobic stress or ethanol. The initial RQ was 1.4–1.6 and decreased to 1.0–1.2 within 30 min, independent of the sugar concentration in the assay. Again, the decrease was caused by a reduction in CO_2 formation. The oxygen consumption remained nearly constant for the duration of the assay.

It can be concluded that the respiration activity either does not change or is slightly activated during oxygen limitation. The respiratory chain is not repressed in the absence of oxygen. In contrast, fermentation is rapidly inactivated at high oxygen concentrations. The rates of both O_2 consumption and of CO_2 formation were

lower if a sugar concentration of 7.4% was used. This was probably because of substrate inhibition of the uptake *(20,21)* or osmotic effects of the high sugar concentrations.

Induction of Enzyme Activities

Activity of PDH

The specific activities of PDH ranged from 0.1–0.5 U/mg and, therefore, were comparatively higher than in *S. cerevisiae (15,22)*, although the measurements of the activity of the *S. cerevisiae* PDH were achieved after a step of purification. The same activities were found with the carbon sources glucose, xylose, or ethanol, and at all levels of oxygen tension tested. These results show that the PDH genes are expressed constitutively in *P. stipitis*. The differences in the specific activities may be caused by a degradation of the multienzyme complex owing to strong protease activities in the crude extract, even though protease inhibitors and mercaptoethanol were added to prevent protein degradations.

Activities of PDC, ADH, and AldDH

In *S. cerevisiae*, addition of glucose to cells grown in ethanol leads to an induction of the PDC *(23,24)*. We compared the activity of PDC in *P. stipitis* cells grown with 2% ethanol and after the addition of 3% glucose. No effect on the activity of PDC by addition of glucose was found within 4 h, and the activity values remained <0.05 U/mg protein.

We tested the induction of the enzyme activities after an oxygen tension shift from aerobic to anaerobic conditions in cells grown on 3% glucose. Figure 1 shows the typical profiles of the activities of PDC, ADH, and AldDH after such a shift. After a lag, the activity of PDC was enhanced 10-fold, that of ADH around 20-fold, and that of aldehyde dehydrogenase 10-fold of the aerobic activity.

To find out whether the induction is an effect resulting from the low oxygen tension or its difference, we tested the activation of the enzymes after a shift from 80 to 20%. After the shift, aeration was further necessary to keep the oxygen tension constant, as respiration occurred. We found a remarkably rapid activation of the enzymes directly after the shift (Fig. 2). This rapid activation of the three enzymes occurred within the first 30 min. The activation of PDC and ADH lasted up to 4 h. Thereafter, the activities of the enzymes decreased. Inactivation of PDC occurred rapidly, indicating perhaps some type of programmed inactivation. An activation of AldDH was only found after the first half hour. Then its activity decreased and reached its initial value within 8 h. We also investigated the production of ethanol, and we found a small amount of ethanol 2–8 h after the shift with maximum rates of 0.1 g/g dry wt and hour.

Figure 3 shows the activation of the three enzymes after the oxygen tension was shifted from 80 to 0% in cells grown on xylose. An induction of the three enzymes also occurred. However, the induction speed of PDC and ADH was slower than in cells grown on glucose, and the activities that were reached after 8 h were only the half of those of cells grown on glucose. The activation of AldDH was similar to that of cells grown on glucose.

Concentrations of Glycolytic Metabolites

To characterize the induction mechanism, we investigated the changes in the intracellular concentrations of the glycolytic metabolites after an addition of glucose

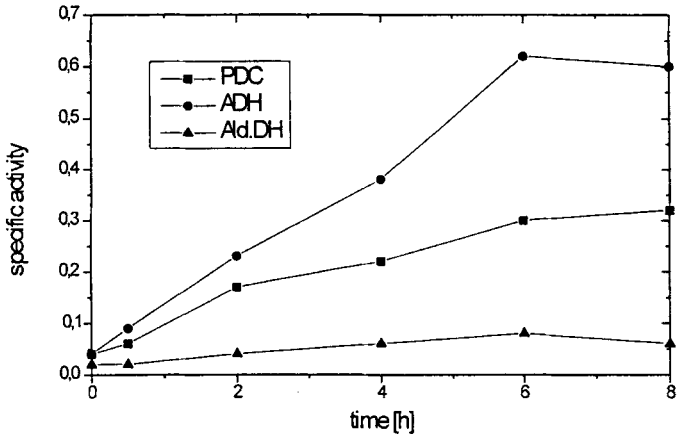

Fig. 3. Specific activities of PDC, ADH, and AldDH of glucose growing cells after a shift of the oxygen tension from 80 to 0%.

to ethanol grown cells, and after an oxygen tension shift with glucose or xylose as carbon source.

Table 1 shows the results from cells grown on ethanol and such cells 2 h after an addition of 3% glucose. Most of the glycolytic metabolites behaved as in *S. cerevisiae* induced with galactose. The concentration of glucose-6-P increased by the same magnitude as in *S. cerevisiae* induced by glucose. However, in *S. cerevisiae*, fermentation is induced after addition of glucose or galactose *(11,25)*.

No significant change in the concentration of glycolytic metabolites, except for pyruvate, was induced by an oxygen tension shift from 80 to 0% in glucose grown cells (Table 2). A threefold increase in the concentration of pyruvate was induced within 30 min by the shift. Thereafter, its concentration decreased to a value about twofold that of the initial level. Similar concentrations of the glycolytic metabolites were measured after a shift of the oxygen tension from 80 to 20%. Glucose-6-phosphate decreased within the first 30 min but after 4 and 8 h, the initial value was nearly reached. The concentration of pyruvate increased as in the shift to 0%. A decrease in the further course of time was also found, but it was not as large as with the shift to anaerobic conditions.

Xylose grown cells differed in their changes of the glycolytic metabolites from cells grown on glucose (Table 3). The concentration of pyruvate marginally decreased in 8 h. Only in the concentrations of glucose-6-phosphate and fructose-6-phosphate was an increase detected. The concentration of pyruvate was found to be much higher in cells grown on glucose or xylose after the shift than in cells grown in ethanol after addition of glucose (Tables 1–3). Moreover, cells grown aerobically with xylose were found to have a higher intracellular concentration of pyruvate than cells grown aerobically with glucose (Tables 2 and 3).

Kinetic Properties of PDC and PDH

We further tested some kinetic properties of PDC and PDH in crude cell extracts. PDC had a Michaelis constant for pyruvate of about $10^{-3}M$, which is nearly in accordance with that of *S. cerevisiae (26)*. The Hill constant 1.4–1.8 was also similar to *S. cerevisiae (26,27)*. The V_{max} in the crude extracts was found to be 0.8–1.1 U/mg

Table 1
Intracellular Concentration of Glycolytic Metabolites (nmol/mg [dry wt]) in Ethanol Grown Cells and of Ethanol Grown Cells 2 h After Addition of Glucose[a]

Growth conditions	G-6-P	F-6-P	FDP	Triose phosphate	3-PG	2-PG	PEP	Pyruvate
Ethanol	1.1	0.5	0.1	0.2	0	2.4	0.4	0.7
Glucose	3.4	0.8	0.2	0.2	0	1.1	0.4	2.3

[a]The results are averages of four experiments. G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, FDP: fructose-1.6.-di-phosphate, 3-PG: 3-phospho-glycerate, 2-PG: 2-phospho-glycerate, PEP: phospho-enol-pyruvate.

Table 2
Time-Course of the Intracellular Concentration of Glycolytic Metabolites (nmol/mg [dry wt]) of Glucose Growing Cells After a Shift of the Oxygen Tension[a]

Time, h	0	0.5		4		8	
O_2	80	0	20	0	20	0	20
G-6-P	5.7	2.1	5.5	4.2	4.7	5.0	4.6
F-6-P	1.4	0.4	1.3	1.0	1.1	0.6	1.0
FDP	2.0	1.0	2.6	1.4	1.1	1.0	1.0
Triose-P	1.0	1.8	0.6	1.9	0.6	1.8	0.7
3-PG	0.6	1.4	1.3	1.6	0.8	1.1	0.5
2-PG	1.0	1.9	0.4	4.0	0.2	2.8	0.7
PEP	0	2.6	1.5	6.4	0	3.4	0.2
Pyruvate	8.8	23.7	19.2	13.0	15.4	14.2	18.9

[a]The oxygen tension at the start was 80% and then shifted to either 0 or 20%. The concentrations of the listed metabolites at various times are listed under 0 (0% O_2) or 20 (20% O_2). The results are averages of three experiments.

Table 3
Intracellular Concentration of Glycolytic Metabolites (nmol/mg [dry wt]) of Xylose Growing Cells After a Shift of the Oxygen Tension from 80 to 0%[a]

Time, h	0	0.5	4	8
G-6-P	1.6	2.3	2.7	3.8
F-6-P	0.4	0.6	1.8	1.0
FDP	3.5	3.8	3.6	3.5
Triose-P	1.2	1.0	1.5	1.4
3-PG	0	0	0	0
2-PG	0.5	0.2	0.4	0.5
PEP	0.8	0.8	0.8	0.8
Pyruvate	15.2	15.1	9.0	13.7

[a]The results are averages of three experiments.

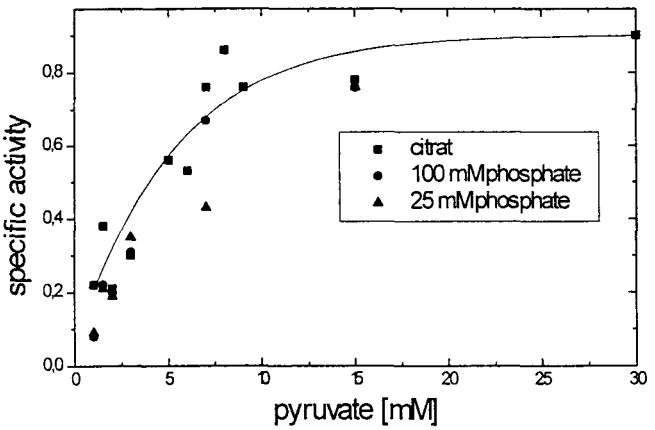

Fig. 4. Kinetic behavior of PDC of *P. stipitis* in phosphate or citrate buffer.

protein. However, no inhibition by phosphate was found. Figure 4 shows the typical behavior of PDC in semiaerobically grown cells.

The Hill constant of PDH with regard to pyruvate was approx 1.0, and the Lineweaver-Burt plot was always linear (results not shown). Therefore, this enzyme was concluded to be a Michaelis-Menten enzyme, as has also been described for *S. cerevisiae (22)*. The Michaelis constant for pyruvate was about $10^{-4}M$, which corresponds to *S. cerevisiae (15)*. NADH was found to inhibit the PDH (results not shown).

DISCUSSION

In *P. stipitis*, the activity of the respiratory chain is not repressed as a result of high concentrations of fermentable sugars or oxygen limitation. Moreover, high concentrations of fermentable sugars do not activate its fermentative pathways. This is in contrast to *S. cerevisiae*, where respiration is repressed and fermentation is activated by both high concentration of glucose and limitation of oxygen *(23,28,29)*.

In *S. cerevisiae* the induction of PDC is well investigated. At least, two different glycolytic signals trigger this induction. The main signal originates from an increase of metabolites of the lower part of glycolysis, including the concentration of pyruvate *(11)*. No induction of PDC was found after addition of glucose to cells grown in ethanol. Nevertheless, the intracellular concentrations of pyruvate behaved similarly to *S. cerevisiae* induced with galactose. Intracellular glucose-6-P was enhanced as in *S. cerevisiae* induced with glucose *(11)*.

PDC was induced in glucose and xylose grown *P. stipitis* cells as a reaction to a shift in the oxygen tension. The concentration of pyruvate increased significantly in glucose grown cells after the shift. However, in xylose grown cells, no increase in the concentration of pyruvate was found under similar conditions. Therefore, we conclude that in *P. stipitis* the main signal for the activation of PDC does not originate from the glycolytic pathway.

The accumulation of pyruvate could act as a minor signal resulting in a stronger induction of PDC in cells grown on glucose. This minor signal perhaps depends on reaching a certain critical concentration of pyruvate. No activation of PDC was found in ethanol grown cells induced with glucose, although the concentration of pyruvate was increased almost three times the initial value. The crucial concentration of pyru-

vate is estimated to be more than 15 nmol/mg dry wt because this concentration was found in aerobic xylose grown cells without any activation of PDC.

These results demonstrate that the mechanism of the induction of PDC in *P. stipitis* is totally different to that of *S. cerevisiae*.

Boles et al. *(11)* detected in *S. cerevisiae* a minor signal derived from the upper part of glycolysis. We found an increase in glucose-6-phosphate and fructose-6-phosphate in both ethanol grown cells induced with glucose and xylose grown cells induced by an oxygen tension shift. This implies that the upper part of glycolysis does not play a role in the regulation of PDC in *P. stipitis*.

The character of the main signal that triggers the PDC activity remains unclear. It was shown, however, from the oxygen shift experiments, that this signal is tightly coupled with the oxygen tension in the medium. Furthermore, the reaction of the cells to the 80–20% shift demonstrates that the signal detects a difference in the oxygen tension rather than a certain low O_2 concentration. Permanent cultivation at 20% oxygen tension does not lead to permanent ethanol formation *(3)*. However, in this study, a transient induction of PDC, ADH, and AldDH was observed after the shift. The presence of oxygen or respiration activity seems to cause a very quick induction of fermentation. The activities of the three measured enzymes were enhanced within 30 min up five times their initial value, which is a faster activation than was described for glycolytic enzymes of *S. cerevisiae (30)*.

ADH and aldehyde dehydrogenase could be regulated in *P. stipitis* by the same O_2-dependent mechanism of PDC. However, acetaldehyde is also a candidate as an inducer of both enzymes. Acetaldehyde has been shown to induce ADH I and AldDH of *Aspergillus nidulans (31)*. The function of AldDH in anaerobiosis may be to prevent an accumulation of acetaldehyde, which is known to be toxic (for a review, *see 32*). However, at anaerobic conditions, the cells have to tolerate the formation of NADH and NADPH by the AldDH reaction *(33)* and, therefore, possibly the generation of a redox imbalance.

Our data demonstrate that, in *P. stipitis* under aerobic conditions, all pyruvate is metabolized via the PDH reaction because PDC is not active. Under oxygen limitation, PDC is induced and the fermentative pathway becomes available for the cell. The activity of PDH and its affinity for pyruvate are relatively high. Initially, this competitive advantage provides the respiratory pathway with pyruvate. However, oxygen limitation is also connected with an increase of intracellular NADH. This high NADH concentration will now inhibit PDH and, therefore, cause the preferential channeling of pyruvate into the fermentative pathway.

Moreover, Skoog and Hahn-Hägerdal *(10)* demonstrated a strong decrease in the intracellular pyruvate concentration after a lengthy time of oxygen limitation. This may be owing to an inhibition of an early step of glycolysis, perhaps the sugar uptake *(2)*. At these low concentrations of pyruvate, however, the velocity of the PDC reaction is slow because of the allosteric kinetics of this enzyme. Therefore, also the ethanol formation rate of *P. stipitis* is low.

The PDC induction signal functions independently of the activities in the glycolytic pathway. It enables the activation of the enzyme in spite of the low glycolytic flux. On the other hand, after a shift to aerobic conditions, the cells are able to switch quickly to the respiratory mode of metabolism because the PDH and the respiratory chain are constitutively expressed, and the inhibition of PDH by NADH is abolished.

The peculiarities of the fermentation regulation mechanism have to be taken into account when manipulating strains to improve their ethanol production. Limi-

tation of oxygen leads to an activation of PDC and ADH, but also to a significant decrease in the glycolytic flux *(10)*. The mechanism of this phenomenon is unknown. Furthermore, *P. stipitis* needs a certain low aeration rate for growth *(3)*.

Ethanol production under aerobic conditions could circumvent the inactivation of the early stages of glycolysis and the requirement for a regulation of the oxygen transfer rate. Such aerobic ethanol production could be achieved by overexpression of *S. cerevisiae* PDC and ADH in *P. stipitis*. However, the PDC of both organisms differ with regard to their inhibition by phosphate. These differences may be disadvantageous during expression of the *S. cerevisiae* PDC in *P. stipitis*.

Furthermore, the aerobic inactivation of PDC has to be taken into account because the inactivation may lead to futile cycles of production and destruction of the enzyme. Moreover, a greater amount of the substrate may be respired, leading to a decrease in the ethanol yield, owing to the high activity of the PDH.

Our results demonstrated large differences between *S. cerevisiae* and *P. stipitis* in the regulation of fermentation and respiration. The model organism of yeast physiology, *S. cerevisiae*, is not comparable in many cases with the physiology of *P. stipitis*. Therefore, improving strains for ethanol production also needs further investigations into the physiology of *P. stipitis*. This knowledge may open up new ways to produce ethanol from xylose and for the understanding of the physiology of Crabtree-negative yeasts.

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